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Molecular Mechanisms in Cell-Mediated Cytotoxicity

Meeting Review

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Intense research has been devoted over the past two decades to the elucidation of the precise molecular mechanisms by which cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells recognize and kill target cells. Both issues have been recently discussed at the EMBO Workshop on Cell-Mediated Cytotoxicity held in Kerkrade, The Netherlands, on April 5–9, 1997, organized by R. L. H. Bolhuis and C. J. M. Melief.

The Two Major Pathways of Cell-Mediated Cytotoxicity

Recent studies have demonstrated that two independent mechanisms account for the cytotoxicity mediated by CTLs (Kagi et al., 1994; Lowin et al., 1994): the first is consequent to the secretion of their characteristic electron-dense cytoplasmic granules whereas the second is represented by a nonsecretory pathway based on the interaction of CD95L (Fas ligand) with the apoptosis-inducer CD95 (Fas) molecule expressed on target cells. So far, the mechanism by which NK cells mediate lysis of NK-susceptible target cells appears to be mostly, if not exclusively, based on granule exocytosis.

The initiation of apoptosis by granule exocytosis is the result of action of two types of molecules released into the extracellular space after contact between effector and target cells: the pore-forming perforin and the lymphocyte-specific granule serine esterase granzyme B (GraB). These molecules interact in the target cells to reproduce all of the features of CTL or NK-induced apoptosis. These include nuclear changes such as chromatin condensation and finally DNA fragmentation.

The mechanism of entry into target cells of GraB and the mechanism by which it induces nuclear damage has been investigated by several groups. Thus, C. Froelich (Northwestern University, Evanston, Illinois) C. Bleackley (University of Alberta, Edmonton, Canada), J. Trapani (Austin Research Institute, Victoria, Australia), and P. Henkart (National Cancer Institute, NIH, Bethesda, Maryland) studied in detail the respective roles of GraB and perforin (Froelich et al., 1996; Jans et al., 1996; Shi et al., 1997). It has been shown that GraB is endocytosed by target cells independently of perforin, possibly through saturable high affinity cell surface binding sites. In the absence of added perforin, GraB shows a cytoplasmic localization. On the other hand, when perforin is added (and presumably enters cells, through an as yet undefined mechanism) in about half of the cells, GraB relocalizes to the nucleus. These cells that show GraB relocalization undergo apoptotic death.

The activation of members of cystein protease interleukin-1β converting enzyme (ICE) family, now called the caspase family (Alnemri et al., 1996), is believed to be required for cell death, at least as to its nuclear manifestations. GraB can cleave and thus activate caspases, leading to cell death. This pathway was examined by C. Bleackley and colleagues, who showed GraB–induced caspase 3 activation and its further processing in target cells (Darmon et al., 1995). However, the possibility that this occurs via an upstream caspase was suggested by J. P. Medema (German Cancer Center, Heidelberg, Germany) and P. Krammer (Inst. fur Immunologie und Genetik, Heidelberg, Germany), who demonstrated that caspase inhibitors that do not block caspase 3 do block its processing induced by CTL. Further results led them to conclude that the caspase FLICE (see below) is a likely substrate for GraB. P. Henkart and colleagues presented similar data showing that GraB can process procaspases leading to their proteolytic activation and to apoptotic nuclear damage (Sarin et al., 1997). However, they also showed that caspase inhibitors block nuclear relocalization and DNA fragmentation but not ^{51}Cr release, strongly suggesting that GraB can also cleave a noncaspase substrate thereby leading to membrane damage. There might well be additional functional molecules in the granules beyond just perforin and GraB. Indeed, Trapani and colleagues showed that the overexpression of Bcl-2 protects cells subjected to perforin and GraB from DNA fragmentation but not cells subjected to cytotoxic cells or to purified granules. Importantly, M. Simon and colleagues (Max-Planck-Institut, Freiburg, Germany) showed that although in vitro– or ex vivo–derived CTL and NK cells (Ebnet et al., 1995) from double knock out GraA$^{-/-}$ GraB$^{-/-}$ mice displayed reduced granule-mediated cytotoxicity in terms of nuclear lesions, they could still induce ^{51}Cr release. This strongly suggests that release of ^{51}Cr can either be mediated by perforin alone, or involves some other granule component as well, possibly another granzyme.

As to the major Fas-based mechanism of cytotoxicity, it is now clear that oligomerization, probably trimerization, of CD95 is necessary to attract intracellularly preformed signaling molecules. The cytoplasmic tail of Fas contains a motif called the “death domain.” This domain can interact with the corresponding death domain of the adaptor molecule Fas-associated protein with death domain (FADD), thus recruiting FADD to the plasma membrane. FADD, in turn, interacts with the caspase FLICE through “death effector domains.” These domains are located at the amino terminus of FLICE and at the carboxyl terminus of FADD. This interaction leads to the assembly of the receptor-associated death-inducing signaling complex (DISC). The DISC-bound FLICE induces the proteolytic activation of other members of the caspase family resulting ultimately in apoptosis (Boldin et al., 1996; Muzio et al., 1996).

A new family of virus-encoded inhibitors termed viral FLICE-inhibitory proteins (vFLIPs) that interferes with apoptosis signaled via the CD95 death receptor was described (J. Tschopp, University of Lausanne, Switzerland). These inhibitors, which are found in particular in

a series of γ-herpes viruses, allow the virus to undergo replication before the host cells finally succumb. The vFLIPs are characterized by two death-effector domains which, similar to FLICE, are able to interact with FADD, thus competing for the recruitment of FLICE induced by the oligomerization of the CD95 receptor (Thome et al., 1997).

Cell death signaling pathways related to the Fas and TNF. R1 pathways also exist in invertebrates. A molecule called "reaper" was shown by H. Steller and his colleagues (White et al., 1994) to be necessary and sufficient to induce cell death in Drosophila during development. Homology was found between the death domains and reaper (Golstein et al., 1995). Some periodicity, such as several successive leucine residues, separated by 7 residues, was detected both in death domains of TNF-R1 and in reaper. Amino acid alignment showed that the middle third of reaper was more homologous to the death domains of TNF-R1 and DR3 (Chinnaiyan et al., 1996) than to the death domains of Fas. Interestingly, the former are bound by TNF-R1-associated protein with death domain (TRADD), the latter by FADD. Thus, both the degree of homology and the connecting molecules are consistent with the existence of subfamilies of death domains. Reaper would be evolutionarily closer to one of these subfamilies (P. Golstein, INSERM-CNRS, Marseille-Luminy, France).

Studies using gene knockout animals (E. R. Podack, University of Miami, Florida) indicated that mice deficient for both perforin and Fas ligand expression died of severe pancreatitis, with atrophic uterus and ovary infiltrated with cells from a particular T cell subpopulation. This might be interpreted as a requirement for both or either the Fas or the perforin pathway for the elimination of antigen-presenting cells; thus when the latter are not eliminated, persistent antigen presentation may lead to continuous T cell activation and tissue damage (autoreactive T cells?).

A novel class of Fas-activated serine/threonine kinases (FASTs) that phosphorylate the RNA binding protein TIA-1 during stress-induced apoptosis has been described by P. Anderson (Dana Farber Cancer Institute, Boston, Massachusetts). TIA-1 is a component of a signaling cascade that may regulate entry into apoptosis (Taupin et al., 1995). TIA-1 binds to oligopyrimidine tracts found in the 5′ untranslated regions of mRNAs subject to stress-induced translational arrest. Some of these mRNAs encode ribosomal subunits and survival factors such as IGF-II, hence proteins that are essential for cell growth. In response to stress, TIA-1 accumulates in the cytoplasm where it aggregates with free and ER-associated ribosomes at "stress bodies." The recruitment of selected Bcl-2 family members to these aggregates has been postulated to regulate entry into apoptosis.

MHC-Class I–Specific NK Cell Receptors

In recent years, the molecular mechanisms by which NK cells lyse or fail to lyse target cells have been elucidated. NK cells express receptors (NKR) that recognize MHC class I molecules on target cells. The interaction between NKR and MHC class I leads to inhibition of cytolytic activity thus preventing lysis of normal, MHC class I positive, target cells. This mechanism was predicted by the "missing self hypothesis" (Ljunggren and Karre, 1990). Different types of inhibitory receptors specific for groups of HLA-C (p58.1 and p58.2), HLA-B (p70), or HLA-A (p70–140) alleles have been molecularly identified and cloned (reviewed by Moretta et al., 1996). They belong to the Ig superfamily and are characterized by two or three extracellular domains. These receptors are distributed in a clonal fashion and each NK cell expresses at least one inhibitory NKR for self HLA–class I alleles. In addition, activating forms of HLA-C-specific receptors have been described (p50.1, p50.2, and p50.3) that differ from the corresponding inhibitory receptors in their transmembrane and cytoplasmic portions. More recently, a second class of MHC class I–specific receptors has been demonstrated in human NK cells (Moretta et al., 1994; Sivori et al., 1996; Lazetic et al., 1996). These NKR display a broad specificity for different HLA-class I molecules although they appear to bind preferentially to certain groups of HLA-alleles (those belonging to the Bw6 supertypic specificity) and poorly to others (Bw4 alleles). They are formed by CD94 molecules that associate with different members of the NKG2 molecular family to form either inhibitory (CD94/NKG2A) or activating NKR (CD94/NKG2C) (A. Moretta, University of Brescia, Italy). Both CD94 and NKG2A molecules are type 2 transmembrane proteins belonging to the C-type lectin superfamily (reviewed by Lopez-Botet et al., 1997). Remarkably, both NKG2A and inhibitory NKR of the Ig superfamily are characterized by intracytoplasmic immunoreceptor tyrosine based inhibition motifs (ITIM), which are essential for SHP-1 phosphatase activation and induction of inhibitory signals (reviewed by Rénard et al., 1997). Because of the molecular structure, the broad specificity and the functional property, CD94/NKG2 complexes could represent the human counterpart of the murine Ly49 family of MHC-class I-specific NK receptors. In view of the recent evolution of the HLA-class I system, it is likely that those NKR belonging to the Ig superfamily have evolved more recently than C-type lectin NKR to adapt to the polymorphism emerging in the HLA class I system (reviewed by Valiante et al., 1997). This receptor strategy allows the NK cell system to sense even single allelic loss as it may occur in tumor or virally infected cells. In this context, CD94/NKG2 complexes would represent an ancestral type of MHC-class I recognition strategy.

Inhibitory NKR are also expressed on a small subset of human T lymphocytes (reviewed by Moretta et al., 1996). NKR^+ T cells have been detected in peripheral blood, spleen, tonsil, and lymph nodes but not in thymus and cord blood. Surface marker analysis has revealed that NKR^+ T cells always express a memory phenotype; moreover, in several normal donors analyzed, they are characterized by a skewed TCRVβ repertoire. Crosslinking of NKR leads to inhibition of different TCR-dependent and TCR-independent (e.g., NK-like cytotoxicity) T cell functions. The inhibitory effect exerted on TCR-mediated functions has important implications. Thus, the expression of inhibitory NKR may result in functional impairment of specific CTLs. Preliminary data would suggest that this phenomenon may occur in tumor (Ikeda et al. 1997) or virally infected patients (L. Moretta, University of Genoa, Italy). The fact that antigen-specific

CTLs simultaneously express TCR and NKR, both recognizing HLA-class I molecules but mediating opposite signals, offers new perspectives in our appreciation of the regulation of T cell responses and provides new clues for understanding the immunopathologic events involved in certain diseases. Data were also presented that are compatible with the existence of additional, still unknown, inhibitory receptors in alloantigen-stimulated T cells. These cells were inhibited in their cytolytic function by a set of HLA-C alleles corresponding to those recognized by the p58.2 receptors; however, they did not react with the presently available anti-p58.2 MAbs (K. Falk, University of Munich, Germany).

Recent studies indicated that the nonclassical HLA-class I molecule HLA-G, which is the only class I molecule expressed by trophoblast cells, is recognized by inhibitory receptors expressed on human NK cells (Pazmany et al., 1996; Munz et al., 1997). This issue has been discussed by H. G. Rammensee (University of Tubingen, Germany) who presented data suggesting that the receptors involved in HLA-G recognition may be related to the HLA-Bw4-specific p70 NKR (or NKAT-3). These data are in conflict with those reported by J. Stromінger's group (Pazmany et al. 1996) showing that HLA-G recognition is mediated by the HLA-C-specific p58 receptors. Further studies are required to elucidate the precise contribution of the various NKR to HLA-G recognition since other groups have recently shown that HLA-G is recognized by the CD94/NKG2A complex and not by p58 or p70 receptors (Pende et al., 1997; Perez-Villar et al., 1997).

The need for inhibitory NKR to prevent NK-mediated lysis of self cells implies the existence of one or more activating receptors that interact with ligand(s) expressed on target cells. However, limited information is available on the surface receptors that are involved in NK cell triggering. Thus the search for new molecules capable of triggering the NK-mediated cytotoxicity is now becoming crucial for defining the molecular mechanisms involved in NK cell activation/inactivation. In this context, a new molecule termed p46, has been identified that induces strong NK cell activation upon cross-linking by specific MAbs (S. Sivori, University of Genoa, Italy). The p46 molecule is selectively expressed on all human NK cells, resting or activated, and the levels of cytolytic activity of different NK clones against HLA-negative targets correlates with the number of p46 molecules expressed at their cell surface. p46 is clearly distinguishable from p50 molecules (i.e., the HLA-C-specific activating receptors) because of its molecular size, cell distribution, and distinct specificity. It may represent a receptor for non-HLA ligands expressed on both HLA^+ and HLA^- target cells. The precise definition of the p46 ligand will be possible upon cloning of this new receptor.

In mice, all the MHC class I-specific receptors characterized so far belong to the Ly49 molecular family (reviewed by Yokoyama and Seaman, 1993). Most of them are inhibitory receptors including Ly49A specific for D^d and D^K, Ly49C specific for K^b (and some $H\text{-}2^d$) and Ly49G2 specific for D^d and L^d. Analysis of the peptide dependency in the NKR-mediated recognition of MHC molecules revealed that Ly49C-mediated recognition is strictly peptide-dependent. Indeed, studies employing TAP-deficient RMA-S target cells showed that "empty" class I molecules do not protect cells from $Ly49C^+$ NK cells. On the other hand, the same cells are protected after incubation in the presence of peptides binding to K^b. This is in line with previous experiments on the Ly49A receptors displaying a similar peptide-dependency (Correa and Raulet, 1995). Similar to the human NKR, a dichotomy of function between the various members of the Ly49 family has been demonstrated also in mice. In particular, cross-linking of Ly49A or Ly49C resulted in thyrosine phosphorylation of the receptors while this was not observed after cross-linking of Ly49D (J. Ortaldo, National Cancer Institute, Frederick, Maryland). These results are consistent with both the known structural and functional characteristics of these Ly49 molecules, in that Ly49A, C, and G2 have ITIM motifs containing phosphorylation sites while Ly49D does not contain this inhibitory motif. Therefore, despite significant divergence in their extracellular domains, human p58/p50 receptors and murine Ly49 receptors are likely to share common signaling properties (reviewed by Rénard et al., 1997).

Viral Strategies to Elude CTL or NK Cell-Mediated MHC Recognition

It is well known that T lymphocytes, primarily MHC class I-restricted $CD8^+$ CTL, play a major role in the control of virus infections. Some DNA viruses, on the other hand, have evolved strategies to interfere with the process of MHC/viral peptide presentation to CTLs (reviewed by Spriggs, 1996). The recent understanding of the mechanisms involved in antigen presentation made possible the unraveling of several of the molecular mechanisms by which viruses interfere with MHC class I antigen presentation. However, while downregulation of cell surface expression of MHC class I molecules (the so-called "stealth strategy") will prevent the CTL-mediated lysis of infected cells, it should render them susceptible to NK-mediated lysis (Karre and Welsh, 1997) (see below).

The first known example of viral protein interfering with MHC class I pathway is the E19 protein of adenovirus (Andersson et al., 1985). Since E19 molecule interacts with MHC class I molecules at the ER level, class I molecules do not reach the cell surface, but they are retrieved from the Golgi together with E19 molecules. Not all the HLA-class I alleles bind equally well to E19, which therefore preferentially inhibits the expression of certain alleles (thus rendering cells susceptible to lysis by NK cell subsets expressing appropriate NKR). Other viruses that inhibit the MHC class I expression include members of the Herpes virus family. For example, the Herpes simplex (HSV) encoded ICP47 molecule (York et al., 1994) binds to TAP1 and TAP2 in the cytosol and prevents the TAP-mediated binding and transport of peptides. The human cytomegalovirus (HCMV) employs different strategies to inhibit HLA class I expression (Fruh et al., 1997). The US6 glycoprotein resides in the ER and interferes with the peptide translocation, but not with their binding to TAP molecules. US2 and US11 (Wiertz et al., 1996) prevent the HLA class I heavy chains from leaving the ER compartment and shuttle them back into the cytosol where they are degraded by proteasomes. US3 protein (Ahn et al., 1996) is expressed very early during HCMV infection, as early as virus-encoded

transcription factors, binds to class I molecules and prevents their export to the surface. Consequently, in the course of HCMV infection, HLA-class I molecules are first retained and then degraded, suggesting that US3 represents the first line of viral interference, followed by US11 and, possibly, by other US proteins (U. Koszinowski, Max von Pettenkofer Institut, Munchen, Germany). It is interesting to note that NK cells play an important role in controlling both HSV and HCMV infections. However, a potential mechanism to escape the NK cell recognition can be the expression of a β2-binding protein with a structural homology to MHC class I heavy chain ("decoy strategy"). This protein, termed UL18, also binds peptides (Beck and Barrell, 1988; Fahnestock et al., 1995). Rather than interfering substantially with antigen presentation to T cells, UL18 has been shown to function by protecting HCMV-infected cells against NK cell lysis (reviewed by Reyburn et al., 1997). Recent experiments showed that UL18 transfection into HLA class I negative target cells conferred to these cells resistance to NK-mediated lysis. The broad specificity CD94/NKG2A receptor appears to be responsible for binding UL18. Therefore HCMV has evolved two complementary strategies to avoid identification of infected cells by both CTLs and NK cells. A corollary of the fact that HCMV has developed a mechanism for evading NK-mediated detection is that NK cells must represent an important component of the cell-mediated immune response to viral infection.

Cytotoxic Lymphocytes in Immunotherapy

The development of strategies for the generation of effective populations of antigen-specific CTL in immune responses against tumor or virus-infected cells represents a major goal of studies of both basic and applied immunology (Bolhuis et al., 1996). In this context, Eshhar pioneered the field of constructing chimeric receptors with tumor Ag specificity (Gross and Eshhar, 1992). T lymphocytes expressing chimeric receptors with the extracellular antibody recognition unit, i.e., with antibody-dictated specificity, efficiently lysed target cells expressing the relevant antigen. Originally, V heavy and V light chains, in a single chain configuration, were linked to the signal transducing γ or ζ subunits (Eshhar et al., 1993). In a recent approach, the antibody recognition unit was connected to a CD4-CD8 transmembrane stretch directly to the intracellular Syk protein-tyrosine kinases. Making chimeric receptors with an NDF/Heregulin binding domain (Stancowski et al., 1993), it has been possible to functionally recognize carcinoma cells overexpressing the Erb family of oncogenic receptors. R. L. H. Bolhuis and colleagues (Daniel den Hoed Cancer Center, Rotterdam, The Netherlands) presented data on the construction of a single chain antibody/γ chimeric receptor specific for renal cancer cells. They also achieved efficient retroviral gene transduction in human primary T cells and optimal in vitro expansion of gene-modified T lymphocytes. This work is expected to result in a phase I clinical study using in vitro–expanded gene-modified T cells expressing renal cell cancer (RCC)-specific chimeric receptors for the treatment of metastatic carcinoma. This approach may benefit from the data presented by Drs. D. J. Schendel (Institut fur Immunologie, Munich, Germany) and L. K. Borysiewicz (College of Medicine, University of Wales, U.K.) who described CTL specific for RCC or cervical cancer, respectively. In renal cancer, two types of CTL were isolated, one detecting subtle changes in MHC expression on tumor cells and the other showing MHC-restricted, RCC-specific recognition of common RCC determinants. These CTL families of T cells specifically accumulate at the tumor site and are highly homologous. In cervical cancer, HPV-16- and 18E6/E7-specific CTL (see below) were identified in peripheral blood in low frequency, but in higher frequencies in draining lymph nodes and tumor infiltrating lymphocytes (TIL). R. E. Toes (University of Leiden, The Netherlands) succeeded in generating tumor-specific CTL by in vitro vaccination with recombinant adenoviruses (rAd) encoding tumor-derived CTL epitopes (human adenovirus type 5 early—region 1A (Ad 5E1A), Ad5E1B and human papilloma virus type 16E7 oncoproteins) in a string-of-bead fashion. The synthetic minigene-encoded CTL epitopes are processed and presented to tumor-specific CTL. rAd-immunized mice were protected against otherwise lethal challenge with tumor cells transformed by the relevant oncoproteins.

The possible use of minor histocompatibility antigens as a target for CTL-mediated specific recognition of leukemic cells (Graft versus leukemia [GvL]) has been emphasized. This approach would allow the elimination of the unwanted graft versus host diseases (GvHDs), which represents a frequent and serious complication in bone marrow allograft. This approach, which is based on pulsing dendritic cells with the minor histocompatibility antigen HA-1 peptide, led to the generation of CTLs specific for HA-1-positive EBV transformed B cell lines (R. M. Verdijk, Leiden University Hospital, The Netherlands). The use of viral "stealth" genes (e.g., MCMV-encoded proteins [Thale et al., 1995]) able to interfere with antigen presentation by MHC class I molecules (see above) is currently being investigated in gene therapy in an attempt to avoid recipients' CTL responses to the vector or even to the therapeutic gene itself (W. Brune, Max von Pettenkofer-Institut, Munich, Germany). It appears that stealth genes themselves, outside the viral context, are able to protect cells from CTL-mediated lysis by lowering MHC class I expression. By using an episomally replicating plasmid vector, which allows for high and stable expression levels, cell lines were established that express one of these genes. In contrast to the parental cell lines and mock transfected cells, these cells displayed a virtually absent expression of MHC class I molecules. Transfectants derived from murine tumor cell lines can now be analyzed in established mouse model systems of tumorigenesis where CTLs are known to influence tumor growth. If CTLs are not generated, the next step will aim at the insertion of the therapeutic gene into the same vector in combination with a "stealth" gene. This therapeutic gene is then expected to be nonimmunogenic.

Another important approach in cancer immunotherapy is focalized on the identification of tumor-associated antigens and their peptides, which can be recognized by specific CTL. These studies led to the identification of numerous tumor-associated antigens (Coulie et al., 1994; Ikeda et al., 1997). Importantly, not only the product of mutated genes are recognized, but also antigenic peptides derived from overexpressed normal genes. Of particular relevance is the tumor suppressor protein p53, because it is overexpressed in up

to 50% of all human malignancies. C. Melief (Dept. of Immunohematology, Leiden Hospital, Leiden, The Netherlands) reported on the generation of CTL recognizing a murine wild-type p53 peptide (Ropke et al., 1996) presented by the H-2^b MHC molecule by immunizing *p53* gene deficient ($p53^{-/-}$) mice with syngenic p53-overexpressing tumor cells. Subsequent adoptive transfer of these CTL in tumor-bearing $p53^{+/+}$ nude mice caused complete and permanent tumor eradication. This occurred in the absence of any demonstrable damage to normal tissues. Thus, overexpression of p53 allows CTL to discriminate between tumor cells and normal tissues, moreover widely expressed autologous molecules such as p53 can serve as a target for CTL-mediated immunotherapy of tumors. In order to induce optimal tumor antigen presentation and thus effective immune responses, monocyte-derived dendritic cells (DCs) have been used as APC to induce primary immune responses against melanocyte differentiation antigens such as gp100, Melan A/Mart 1-, or tyrosinase-derived peptides presented by HLA-A2.1. These in vitro–generated CTL were capable of lysing HLA-A2.1-positive tumor cells expressing the appropriate antigen, suggesting that these antigen-loaded DCs could provide useful immunotherapeutic reagents for the induction of CTL responses against autologous tumors (C. G. Figdor, Department of Tumor Immunology, Nijmegen Hospital, Nijmegen, The Netherlands). Finally, evidence is now available on the possibility of inducing peptide-specific down-regulation of T cell responses to intervene in autoimmune diseases or graft rejections. Efficient tolerance induction has been achieved in unprimed animals by intraperitoneal administration of peptides in incomplete Freund's adjuvants. However, in the presence of peptide-specific memory CTL, the same peptide treatment not only abrogates the peptide-specific CTL activity but also has a general immunosuppressive effect as a consequence of severe immunopathological damage in secondary lymphoid organs. Peptide treatments of antigen-primed hosts therefore may have deleterious and undesirable effects, which has to be taken into account for therapeutic interventions (reviewed by Kagi et al., 1996) (H. Hengartner, University Hospital, Zurich, Switzerland).

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